FadD19 of Rhodococcus rhodochrous DSM43269, a Steroid-Coenzyme A Ligase Essential for Degradation of C-24 Branched Sterol Side Chains

Wilbrink, M. H.; Petrusma, M.; Dijkhuizen, L.; van der Geize, R.

Published in:
Applied and environmental microbiology

DOI:
10.1128/AEM.00380-11

Publication date:
2011

Applied and Environmental Microbiology, July 2011, p. 4455–4464 Vol. 77, No. 13
0099-2240/11/$12.00 doi:10.1128/AEM.00380-11

FadD19 of *Rhodococcus rhodochrous* DSM43269, a Steroid-Coenzyme A Ligase Essential for Degradation of C-24 Branched Sterol Side Chains▽†

M. H. Wilbrink, M. Petrusma, L. Dijkhuizen, and R. van der Geize*

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, P.O. Box 14, 9750 AA Haren, Netherlands

Received 19 February 2011/Accepted 2 May 2011

The actinobacterial cholesterol catabolic gene cluster contains a subset of genes that encode β-oxidation enzymes with a putative role in sterol side chain degradation. We investigated the physiological roles of several genes, i.e., *fadD17*, *fadD19*, *fadE26*, *fadE27*, and *ro04690*$_{DSM43269}$, by gene inactivation studies in mutant strain RG32 of *Rhodococcus rhodochrous* DSM43269. Mutant strain RG32 is devoid of 3-ketosteroid 9α-hydroxylase (KSH) activity and was constructed following the identification, cloning, and sequential inactivation of five *kshA* gene homologs in strain DSM43269. We show that mutant strain RG32 is fully blocked in steroid ring degradation but capable of selective sterol side chain degradation. Except for RG32Δ*fadD19*, none of the mutants constructed in RG32 revealed an aberrant phenotype on sterol side chain degradation compared to parent strain RG32. Deletion of *fadD19* in strain RG32 completely blocked side chain degradation of C-24 branched sterols but interestingly not that of cholesterol. The additional inactivation of *fadD17* in mutant RG32Δ*fadD19* also did not affect cholesterol side chain degradation. Heterologously expressed FadD19$_{DSM43269}$ nevertheless was active toward steroid-C$_{26}$-oic acid substrates. Our data identified FadD19 as a steroid-coenzyme A (CoA) ligase with an essential *in vivo* role in the degradation of the side chains of C-24 branched-chain sterols. This paper reports the identification and characterization of a CoA ligase with an *in vivo* role in sterol side chain degradation. The high similarity (67%) between the FadD19$_{DSM43269}$ and FadD19$_{H37Rv}$ enzymes further suggests that FadD19$_{H37Rv}$ has an *in vivo* role in sterol metabolism of *Mycobacterium tuberculosis* H37Rv.

Phytosterols are among the most abundant sterols in nature and are mineralized from decaying plant material by soil bacteria, e.g., *Rhodococcus* strains belonging to the class *Actinobacteria* (37). Microbial degradation of sterol molecules involves elimination of the alkyl side chain and opening of the polycyclic steroid nucleus (Fig. 1) (1, 13, 26–28). The order of these two processes varies between bacterial genera and even between members of the same genus (23).

Prior to their degradation, sterols are actively transported into the actinobacterial cells by the Mce4 steroid transporter (17, 20, 36). Following uptake by *Rhodococcus jostii* RHA1 cells, the cytochrome P450 monooxygenase CYP125 initiates degradation of the sterol side chain by catalyzing hydroxylation of the C-26 or C-27 carbon (Fig. 1A and B, reaction 1) (5, 15, 19, 23). After complete oxidation of the hydroxylated terminal carbon atom to its carboxylic acid intermediate (Fig. 1, compounds III and XI), the sterol side chain is shortened by a process similar to β-oxidation of fatty acids. This process is initiated by an ATP-dependent sterol/steroid-coenzyme A (CoA) ligase (Fig. 1A and B, reaction 3) (6, 27, 28) catalyzing the CoA activation of the C$_{26}$ carboxylic acid intermediates (Fig. 1, compounds III and XI). An ATP-dependent steroid-CoA ligase (65 kDa) from *Mycobacterium* sp. strain NRRL B3805 was purified to near homogeneity and shown to be highly specific toward C$_{26}$ carboxylic sterols (6). The gene encoding this activity remained unknown, however. Thiolytic cleavage results in shortening of the cholesterol side chain in several rounds of β-oxidation via C$_{24}$ (Fig. 1A, compound IV) and C$_{22}$ (Fig. 1A, compounds V and VII) intermediates and concomitant release of propionyl-CoA and acetyl-CoA, respectively. This process has been elucidated at the biochemical level in strains of *Mycobacterium* and *Nocardia* and proceeds differently for β-sitosterol than for cholesterol (Fig. 1) (9, 10, 13, 14, 27–29).

Knowledge of genes involved in microbial degradation of the alkyl sterol side chain is extremely limited. Recently, a wealth of information on sterol catabolism was provided by the identification of a cholesterol catabolic gene cluster in *R. jostii* RHA1, encoding several enzymes with predicted functions in β-oxidation (36). A subset of these genes (i.e., *ro04683* to *ro04694*) is located proximal to *cyp125*, encoding a steroid 26-hydroxylase involved in sterol side chain degradation (5, 15, 19, 23, 36). The aim of our work is to decipher the roles of these genes in sterol metabolism, particularly those genes encoding steps of the β-oxidation cycle of sterol side chain degradation. The strain RHA1 cholesterol transcriptome was used to identify two candidate genes, i.e., *fadD17* (*ro04691*) and *fadD19* (*ro04689*), encoding putative acyl-CoA ligases, which were highly upregulated during growth on cholesterol (Fig. 2A) (36). Previous studies on FadD17 and FadD19 homologs from *Mycobacterium tuberculosis* H37Rv showed that *in vitro* they act as CoA ligases capable of activation of long-chain fatty acids (31). Their *in vivo* physiological roles remained unknown.

* Corresponding author. Mailing address: Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, Netherlands. Phone: 31 (50) 3632153. Fax: 31 (50) 3632154. E-mail: r.van.der.geize@rug.nl.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 20 May 2011.

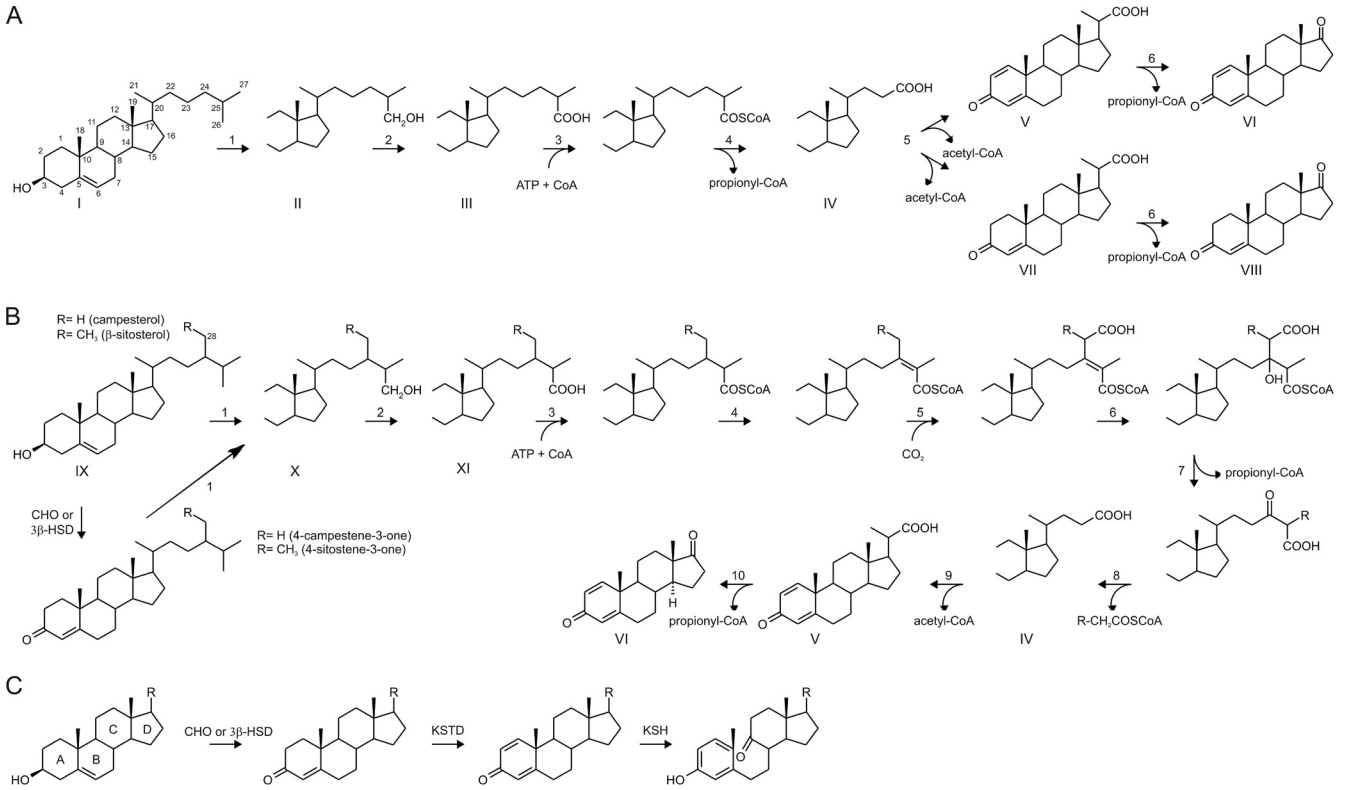

FIG. 1. Schematic overview of the side chain degradation pathways of cholesterol (A), β-sitosterol and campesterol (B), and steroid ring opening (C) in actinobacteria (6, 10, 36). The arrow numbering indicates reaction steps which are explained in the text. Abbreviations: CoA, coenzyme A; CHO, cholesterol oxidase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; KSTD, 3-ketosteroid Δ1-dehydrogenase; KSH, 3-ketosteroid 9α-hydroxylase.

Only a few examples of sterol side chain degradation genes have been reported, including *baiB* of *Eubacterium* sp. strain VPI 12708 and *caiA* of *Pseudomonas* sp. strain Chol1, encoding a CoA ligase and an acyl-CoA dehydrogenase, respectively, involved in cholic acid degradation (4, 12). Also, *fadA5* of *M. tuberculosis* H3Rv was shown to encode a thiolase with a role in cholesterol side chain degradation (18).

Molecular characterization studies on sterol side chain degradation are generally hampered by the lack of a stable, genetically accessible strain with inactivated steroid ring degradation but capable of selective sterol side chain degradation. Interestingly, *Rhodococcus rhodochrous* IFO3338 (=DSM43269) is amenable to genetic manipulation and able to selectively degrade the sterol side chain in the presence of iron chelators, which inhibit 3-ketosteroid 9α-hydroxylase (KSH) activity (2, 23). KSH is a two-component enzyme system, consisting of a terminal oxygenase, KshA, and a ferredoxin-reductase, KshB, and a key enzymatic step in steroid ring opening during microbial steroid catabolism (22, 33, 35). We created a genetically stable, multiple *kshA* gene deletion null

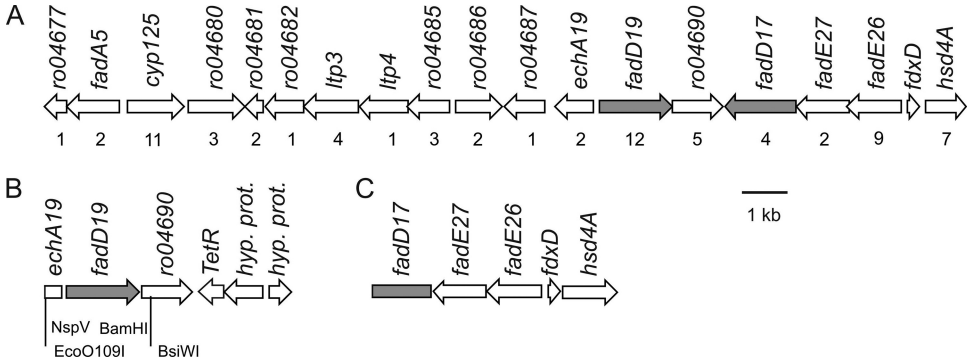

FIG. 2. (A) β-Oxidation gene cluster comprised of *ro04677* to *ro04695* within the cholesterol catabolic gene cluster of *R. jostii* RHA1 (16, 36). The relative fold change of expression previously observed during growth on cholesterol compared to pyruvate is also shown (36). (B and C) Genetic organization of homologs of *ro04688* to *ro04690* and *ro04691* to *ro04695* of *R. rhodochrous* DSM43269, respectively.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference(s)
Strains		
E. coli		
BL21(DE3)	Host for expression of T7-based plasmids	Novagen
DH5α	General host for cloning purposes	Bethesda Research Laboratories
S17-1	Host strain for conjugal mobilization of pK18mobsacB-derived mutagenic plasmids to *Rhodococcus* strains	DSMZ culture collection
Rhodococcus rhodochrous		
DSM43269	Wild-type strain; potent sterol degrader; identical to strain IFO3338	DSMZ culture collection
RG32	Mutant strain of DSM43269 capable of selective sterol side chain degradation; carries 5-fold unmarked *kshA* gene deletion	This study
RG32Δ*ro04690*$_{DSM43269}$	Gene deletion mutant of *ro04690*$_{DSM43269}$ *in R. rhodochrous* strain RG32	This study
RG32Δ*ro04690*$_{DSM43269}$ Δ*fadE26*	Double deletion mutant of *ro04690*$_{DSM43269}$ and *fadE26* in *R. rhodochrous* strain RG32	This study
RG32Δ*ro04690*$_{DSM432690}$ Δ*fadE26* Ω*fadE27*	Gene inactivation mutant of *fadE27* in RG32Δ*ro04690*$_{DSM43269}$ Δ*fadE26*	This study
RG32Δ*fadD19*	Gene deletion mutant of *fadD19* in *R. rhodochrous* strain RG32	This study
RG32 Ω*fadD17*	Gene inactivation mutant of *fadD17* in *R. rhodochrous* strain RG32	This study
RG32Δ*fadD19* Ω*fadD17*	Gene inactivation mutant of *fadD17* in RG32Δ*fadD19*	This study
Plasmids		
pK18mobsacB	Conjugative plasmid for gene mutagenesis in *Rhodococcus*; *aphII sacB oriT* (RP4) *lacZ*	25, 34
pBluescript(II)KS	General *E. coli* cloning vector; *bla lacZ*	Stratagene
pRESQ	*E. coli-Rhodococcus* shuttle vector; *aphII lacZ-ccdB rep* (pMVS301)	35
pZeRO2.1	General cloning vector	Invitrogen
pBs-Pkan	pBluescript(II)KS containing *aphII* promoter region	32
pET15b	T7 promoter-based expression plasmid; *bla lacI*	Novagen
pET15b*fadD19*$_{DSM43269}$	pET15b containing *fadD19*$_{DSM43269}$	This study
pDELBox	pK18mobsacB-derived mutagenic plasmid for deletion of *ro04683* to *ro04694* in RHA1	This study
pDEL*fadD19*$_{DSM43269}$	pK18mobsacB-derived mutagenic plasmid for deletion of *fadD19*$_{DSM43269}$ in RG32	This study
pDEL*fadE26*$_{DSM43269}$	pK18mobsacB-derived mutagenic plasmid for deletion of *fadE26*$_{DSM43269}$ in RG32	This study
pDEL*ro04690*$_{DSM43269}$	pK18mobsacB-derived mutagenic plasmid for deletion of *ro04690*$_{DSM43269}$ in RG32	This study
pΩ*fadE27*$_{DSM43269}$	pK18mobsacB-derived mutagenic plasmid for disruption of *fadE27*$_{DSM43269}$ in RG32	This study
pΩ*fadD17*$_{DSM43269}$	pK18mobsacB-derived mutagenic plasmid for disruption of *fadD17*$_{DSM43269}$ in RG32	This study
pRESQ4690	Genomic library clone DSM43269 carrying *ro04690*$_{DSM43269}$	This study
pRESQ4693	Genomic library clone DSM43269 carrying *fadE26*$_{DSM43269}$	This study
pCOMP*fadD19*$_{DSM43269}$	pRESQ-derived plasmid carrying *fadD19*$_{DSM43269}$ behind the *aphII* promoter for functional complementation of RG32Δ*fadD19*	This study

mutant strain of *R. rhodochrous* DSM43269 (strain RG32) lacking KSH activity. Analogously to the chemical inhibition of strain DSM43269 by iron chelators, mutant strain RG32 was completely blocked in steroid ring degradation and capable of selective sterol side chain degradation, thereby accumulating 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid (1,4-BNC) and 1,4-androstadiene-3,17-dione (ADD) (Fig. 1, compounds V and VI) from sterols. The strain RG32 was used as a tool to assess the role of various genes in sterol side chain degradation. We identified *fadD19* as an essential gene for the degradation of the C-24 branched-chain sterols campesterol and β-sitosterol but not cholesterol. Direct enzyme activity measurement of heterologously produced FadD19 of DSM43269 conclusively identified its function as a steroid-CoA ligase in view of its ability to activate steroid carboxylic acid side chains.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids, and chemicals. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains and *R. rhodochrous* DSM43269 were grown in Luria-Bertani (Sigma, Zwijndrecht, Netherlands) medium at 37°C and 30°C, respectively. Liquid cultures were grown with shaking (220 rpm). Ampicillin (100 μg ml^{-1}), kanamycin (25 μg ml^{-1}), or apramycin (50 μg ml^{-1}) was added to the medium if appropriate after autoclaving. For growth on solid medium, Difco agar (Becton, Le Pont de Claix, France) was added at a final concentration of 1.5% (wt/vol).

Cholesterol, campesterol, and coenzyme A trilithium salt were obtained from Sigma-Aldrich. β-Sitosterol was obtained from Acros Organics. 5-Cholestene-

26-oic acid-3β-ol was synthesized at Schering-Plough, Oss, Netherlands (23). 4-Androstene-3,17-dione (AD), 1,4-androstadiene-3,17-dione (ADD), 23,24-bisnorchol-5-en-22-oic acid-3β-ol, 3-oxo-23,24-bisnorchol-4-en-22-oic acid (4-BNC), 4-cholestene-3-one, 1,4-cholestadiene-3-one, 5-cholenic acid-3β-ol, and 3-oxo-4-cholestene-26-oic acid were obtained from Steraloids. ATP was purchased at Duchefa, Haarlem, Netherlands.

General molecular techniques. Recombinant DNA techniques were performed according to standard protocols (24). DNA-modifying enzymes (restriction enzyme ligases and DNA polymerases) were purchased from Roche (Mannheim, Germany), New England BioLabs (Beverly, MA), or Fermentas (St. Leon-Rot, Germany) and were used according to the manufacturer's protocols. All PCR products were obtained using *Pwo* polymerase (Roche) using standard conditions: 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. DNA fragments were purified from an agarose gel using a Sigma Genelute gel extraction kit.

Gene cloning of four *kshA* homologs of *R. rhodochrous* strain DSM43269 and construction of *kshA* null mutant *R. rhodochrous* strain RG32. We previously reported the cloning, heterologous expression, and characterization of a *kshA* homolog of *R. rhodochrous* DSM43269 (22), that was renamed *kshA4* (see Results). Four additional *kshA* homologs of *R. rhodochrous* DSM43269 were cloned, sequenced, and deleted in DSM43269 as follows. A genomic library of *R. rhodochrous* DSM43269 (22) was introduced by electroporation into Δ*kshA* mutant *Rhodococcus erythropolis* RG2 (35) for functional complementation of growth on AD, resulting in a clone carrying 5.4 kb of insert DNA harboring the *kshA2* homolog of strain DSM43269. A mutagenic plasmid for unmarked *kshA2* gene deletion in strain DSM43269 was constructed as follows. A 2.3-kb BglII DNA fragment containing *kshA2* and its upstream region was ligated into pZeRO (pKSH503). A 1.6-kb BglII/HindIII DNA fragment containing *kshA2* and its downstream region was ligated into pZeRO (pKSH516). A 1.2-kb BamHI/Asp718I fragment of pKSH516 was ligated into BamHI/Asp718I-digested pKSH503, and a 2.5-kb HindIII/SpeI DNA fragment of the resulting construct carrying the *kshA2* deletion was ligated into HindIII/XbaI-digested pK18mobsacB (25), producing pKSH518 used to construct the Δ*kshA2* mutant of *R. rhodochrous*. Next, genomic DNA was isolated from the Δ*kshA2* mutant and used as template for PCR with *kshA* degenerate primers KshAType-F and KshAType-R (33). The obtained 0.3-kb amplicon was cloned and sequenced, identifying the *kshA1* homolog from strain DSM43269. Gene-specific primers for *kshA1* (KshA1Rho-F and -R, Table 2) were developed and used to isolate a clone from the genomic library of *R. rhodochrous* DSM43269 carrying *kshA1*. A mutagenic plasmid for unmarked *kshA1* gene deletion was constructed as follows. A 1.6-kb EcoRI (blunt-ended by Klenow)/Asp718I DNA fragment containing the 5′ part of *kshA1* and its upstream region and a 2.3-kb BglII (blunt-ended by Klenow)/BamHI DNA fragment containing the 3′ part of *kshA1* and its downstream region were ligated stepwise into pBluescript(II)KS. A 3.4-kb XbaI/EcoRI DNA fragment of the resulting construct carrying the *kshA1* deletion was ligated into XbaI/EcoRI-digested pK18mobsacB and used to construct a Δ*kshA1* Δ*kshA2* double gene deletion mutant of the *R. rhodochrous* Δ*kshA2* mutant. Genomic DNA was again isolated from the Δ*kshA1* Δ*kshA2* mutant and used as template for PCR with *kshA* degenerate primers. PCR product was obtained both with primer pair KshAType-F and KshAType-R and with primer pair KshA2-F and KshA2-R (33), the latter pair resulting in a 0.6-kb amplicon containing the *kshA3* homolog of strain DSM43269. Specific *kshA3* primers (KshA3Rho-F and-R, Table 2) were developed and used to isolate a clone (pKSH800) from the genomic library carrying *kshA3*. A mutagenic plasmid for unmarked *kshA3* gene deletion was constructed as follows. A 3.5-kb BamHI fragment of pKSH800 was ligated into BamHI-digested pBluescript(II)KS, which was subsequently digested with BglII and MluI, blunt ended by Klenow treatment, and self ligated. Next, a 1.7-kb BamHI fragment was ligated into BamHI digested pK18mobsacB, rendering the plasmid for *kshA3* gene deletion of the *R. rhodochrous* Δ*kshA1* Δ*kshA2* mutant strain.

A mutagenic plasmid for *kshA4* gene deletion was constructed as follows. A 4.2-kb XbaI/HindIII DNA fragment harboring *kshA4* was subcloned into XbaI/HindIII-digested pZeRO. The resulting plasmid was digested by XhoI, treated with Klenow fragment, and digested with XbaI to give a 4.5-kb fragment harboring pZeRO and the downstream flanking region of *kshA4*. The upstream region of *kshA4* was subsequently ligated as a 1.9-kb fragment liberated following digestion by XhoI, treatment with Klenow fragment, and digestion by XbaI. Next, a 2.8-kb PstI/BamHI fragment was cloned into PstI/BamHI-digested pK18mobsacB, resulting in pKSH631 used for *kshA4* gene deletion to construct Δ*kshA1* Δ*kshA2* Δ*kshA3* Δ*kshA4* mutant strain RG31. Genomic DNA was isolated from mutant strain RG31 and used as template for PCR with *kshA* degenerate primers KshAType-F and KshAType-R (33), resulting in the *kshA5* homolog of DSM43269. Specific *kshA5* primers (KshA5Rho-F and -R, Table 2) were developed to isolate a clone from the genomic library carrying *kshA5*. A 1.5-kb Eco47III fragment carrying the downstream region of *kshA5* was cloned into EcoRV-digested pBluescript(II)KS and subsequently ligated as an EcoRI/Asp718I fragment into EcoRI/Asp718I-digested pBluescript(II)KS carrying a 1.6-kb Asp718I (Klenow-treated) fragment harboring *kshA5* and its upstream region. A 1.05-kb BglII/XhoI fragment of the resulting construct was ligated into BamHI/SalI-digested pK18mobsacB, resulting in pKSH912 used for *kshA5* gene deletion to construct Δ*kshA1* Δ*kshA2* Δ*kshA3* Δ*kshA4* Δ*kshA5* mutant strain *R. rhodochrous* RG32. Genomic DNA was isolated from mutant strain RG32 and used as template for PCR with *kshA* degenerate primers KshAType-F and KshAType-R (33), which did not reveal the presence of additional *kshA* genes.

Screening of a *R. rhodochrous* DSM43269 genomic library for *ro04690* and *fadE26* homologs. Degenerate *ro04690*^{DEG} primers (Table 2) were based on conserved amino acid sequences HTGE(I/V)A(S/T)M (amino acids [aa] 193 to 200) and PLPMPLQ (aa 306 to 312) in Ro04690. Degenerate *fadE26*^{DEG} primers (Table 2) were based on conserved amino acid sequences V(I/V)NGQKMW (aa 153 to 160) and FGGGTNE(V/I) (aa 369 to 376) in FadE26$_{RHA1}$. A genomic library of *R. rhodochrous* DSM43269 (22) was screened by PCR using the *ro04690*^{DEG} and *fadE26*^{DEG} primers for clones containing the full-length *ro04690* and *fadE26* genes, respectively.

Targeted disruption and unmarked gene deletions in *Rhodococcus* strains. Disruption and unmarked gene deletion mutants of *R. rhodochrous* DSM43269 were constructed using the *sacB* counterselection system (34).

Mutagenic plasmids for targeted gene deletion were constructed by PCR amplification of up- and downstream regions of the target genes using the primers listed in Table 2. The obtained amplicons were separately subcloned into EcoRV-digested pBluescript(II)KS. The amplicons were then ligated together using a native restriction site from pBluescript(II)KS (EcoRI or XbaI) and the restriction site that was introduced by PCR, being either BglII or NdeI. The genomic fragment was then isolated by digestion with either EcoRI or XbaI and HindIII and cloned into pK18mobsacB.

A construct for deletion of *fadD19*$_{DSM43269}$ in strain RG32 was made as follows: pRESQ-4690 was digested with EcoO119I and PstI, giving a 3.0-kb fragment, whose 5′ overhang was filled in with Klenow fragment and 3′ overhang was removed by T4 DNA polymerase. Plasmid pK18mobsacB was digested with BamHI and made blunt ended by Klenow fill-in and used for ligation with the blunt-ended 3.0-kb fragment, giving pK18*fadD19*. A 751-bp deletion was introduced by digesting pK18*fadD19* with NspV and BamHI, followed by 5′ overhang fill-in using Klenow fragment and self-ligation, yielding mutagenic plasmid pDEL*fadD19*$_{DSM43269}$.

Mutagenic plasmids for targeted gene disruptions were obtained by PCR amplification of internal fragments of the target genes, using the primers listed in Table 2. The amplicons were subsequently ligated into SmaI-digested pK18mobsacB.

Mutagenic plasmids were transferred to *Escherichia coli* S17-1 by transformation and subsequently mobilized to *R. rhodochrous* DSM43269 by conjugational transfer as described previously (34). All mutants were verified by PCR using specific primers (Table 2) to confirm deletion or disruption of the target gene(s).

Functional complementation of RG32Δ*fadD19*. The *fadD19*$_{DSM43269}$ gene was amplified by PCR using the PCR primers compfadD19-F and compfadD19-R (Table 2). The obtained PCR product of 1,673 bp was digested with Acc65I and ligated into EcoRV/Acc65I-digested pBs-Pkan, yielding plasmid pBs-Pkan-*fadD19*$_{DSM43269}$. The pBs-Pkan-*fadD19*$_{DSM43269}$ plasmid was digested with SpeI/Acc65I to obtain the 2.1-kb Pkan-*fadD19*$_{DSM43269}$ cassette that was subsequently ligated into SpeI/Acc65I-digested pRESQ, resulting in plasmid pCOMP*fadD19*$_{DSM43269}$ that was used to transform electrocompetent cells of RG32Δ*fadD19* as described previously (8).

Whole-cell biotransformation of *R. rhodochrous* RG32-derived mutants and steroid analyses. Cell cultures of *R. rhodochrous* RG32 and derived mutant strains were grown overnight in liquid LB medium at 30°C with shaking (220 rpm) until mid-exponential phase. Stock solutions of sterols were prepared by dissolution in acetone at a concentration of 25 mM and added to cultures at a final concentration of 0.5 mM. After further incubation for 3 days under the same conditions, samples were taken for high-pressure liquid chromatography (HPLC) analysis.

To analyze sterol conversions, 1-ml culture samples were taken and centrifuged (1 min at 16,000 × *g*) and 0.5 ml of supernatant was mixed with 2 ml methanol and filtered prior to analysis by HPLC. HPLC was performed on an Alltima C_{18} column (250 by 4.6 mm; 35°C; Alltech, Deerfield, IL) using a mobile phase consisting of methanol-water-formic acid (80:19:1) at a flow rate of 1 ml min^{-1} with UV detection at 254 nm. Authentic 1,4-androstadiene-3,17-dione and 3-oxo-23,24-chola-1,4-dien-22-oic acid, obtained by incubating authentic 3-oxo-23,24-bisnorchol-4-en-22-oic acid with purified 3-ketosteroid dehydroge-

TABLE 2. List of primers used in this study[a]

No.	PCR amplicon	Size (bp)	Oligonucleotide sequence
1	Genomic library clone carrying *kshA1*	227	KshA1Rho-F, GCCGCTGCAAGAACATCCCGTAC KshA1Rho-R, GATGATCTCGCGGCAGTTGGTGTC
2	Genomic library clone carrying *kshA3*	547	KshA3Rho-F, CGACCTGTCACAGGGCGAGAT KshA3Rho-R, TACGGATTCGGTCTGGTACC
3	Genomic library clone carrying *kshA3*	161	KshA5Rho-F, CGCCTGGACGACCCTCGAACGCA KshA5Rho-R, AGTGGGAGCCCTTGATGCGCAG
4	Internal fragment *ro04690* homolog (screening genomic library)	361	ro04690^{DEG}-F, CACACCGG(C/T)GAG(A/G)T(C/G)GCG(A/T)C(C/G)ATG ro04690^{DEG}-R, TCTGCAGCGGCAT(C/G)(C/G)(C/G)(A/C)AGCGG
5	Internal fragment *fadE26* homolog (screening genomic library)	670	fadE26^{DEG}-F, (C/G)(A/G)TCAACGG(A/C)CAGAAGATGTGG fadE26^{DEG}-R, A(C/T)(C/T)TCGTTGGT(G/T)CCGCCGCCGAA
6	*fadD19*$_{DSM43269}$ gene (heterologous expression in pET15b)	1,679	fadD19exp-F, TCATATGGCCCTAAACATCGCAGACC fadD19exp-R, TAGATCTCTATCCCGTCGCCGCGGCGCCG
7	*fadD19*$_{DSM43269}$ gene (confirmation Δ*fadD19* mutant)	527 (wt, 1,278)	fadD19Contr-F, GTGAAGACCGTCGTGGTGGTAG fadD19Contr-R, GTTGCCGTCGCCGTTGTTGTAG
8	*fadD19*$_{43269}$ gene (complementation Δ*fadD19* mutant)	1,673	COMPfadD19-F, GGCCCTAAACATCGCAGACCTC COMPfadD19-R, CGGTACCCTATCCCGTCGCCGCGGCGCCG
9	Internal fragment *fadD17*$_{DSM3269}$ (disruption construct)	826	fadD17-F, GACATCGCGCTCGCCGATTGCC fadD17-R, TGTAGTAGCCGTCGAACAGACC
10	*fadD17*$_{DSM43269}$ (confirmation *fadD17* disruption)	1,027	pK18Contr-F, AATGCAGCTGGCACGACAGGTT fadD17Contr-R, CGAGCATCCTGCGCGAACTCGG
11	Internal fragment *fadE27*$_{DSM3269}$ (disruption construct)	753	fadE27-F, GCAGCATCGTCGCGGACATC fadE27-R, GGAGCCGAGCAGGAACTCGT
12	*fadE27*$_{DSM43269}$ (confirmation *fadE27* disruption)	1,510	fadE27Contr-F, GCAGCATCGTCGCGGACATC pK18Contr-R, CTGCGTGCAATCCATCTTGTTC
13	Upstream region *ro04690*$_{DSM43269}$ (deletion construct)	1,247	ro04690UP-F, AGCGCCGACGACATCTACATCC ro04690UP-R, TCATATGCGTGAATCCGAAGATCGGATAC
14	Downstream region *ro04690*$_{DSM43269}$ (deletion construct)	1,236	ro04690DOWN-F, TCATATGGAGATCATGGCCGAACTCGTC ro04690DOWN-R, CTGCAGGATCACGGCAACGAC
15	*ro04690*$_{DSM43269}$ gene (confirmation Δ*ro04690* mutant)	129 (wt, 1,113)	ro04690Contr-F, ATGCAGACCGGCCTCAGCAAGA ro04690Contr-R, CTAGCGGGCCTCGTTCAGCCGTTC
16	Upstream region *fadE26*$_{DSM43269}$ (deletion construct)	1,234	fadE26UP-F, TCGAGCAGTTCGGTAACGGTGAG fadE26UP-R, AGATCTCGAGGTCCAGCGCGACATCATC
17	Downstream region *fadE26*$_{DSM43269}$ (deletion construct)	1,230	fadE26DOWN-F, TTAGATCTTTCCTCGCGGAGGGCTTGTTGC fadE26DOWN-R, GGCGAGATAGGCGACCAGATTC
18	*fadE26*$_{DSM43269}$ gene (confirmation Δ*fadE26* mutant)	520 (wt, 1,593)	fadE26Contr-F, GCTTCACGCACGGCGTCCTCAC fadE26Contr-R, GCGGTCCTCGGAGGCATCGAGT

[a] Introduced restriction sites are underlined. wt, wild type.

nase enzyme (Δ1-KSTD1) (11), were used as references to enable quantification of the conversion rates. Steroids were extracted from culture broth using 2 volumes of ethyl acetate. Samples were directly applied onto the same HPLC and detection system as described above, except that the mobile phase consisted of acetonitrile-tetrahydrofuran (75:25) and the flow rate was set at 2 ml min^{-1}. Authentic β-sitosterol (a mixture of β-sitosterol and campesterol) was incubated with cholesterol oxidase (Sigma) to obtain 4-sitostene-3-one and 4-campestene-3-one and used as reference sample.

Production of CoA ligase $FadD19_{DSM43269}$. The *$fadD19_{DSM43269}$* gene was amplified by PCR from genomic DNA of *R. rhodochrous* DSM43269 using primer pair fadD19exp-F and fadD19Exp-R (Table 2). After subcloning of the amplicon in pBluescript(II)KS, the gene was cloned into pET15b using NdeI and BamHI, yielding pET15b*$fadD19_{DSM43269}$*, and introduced into competent *E. coli* BL21(DE3) cells for expression. Overexpression of $FadD19_{DSM43269}$ was obtained by growing 50-ml cultures in LB medium, supplemented with 0.5 M sorbitol, 100 mM ampicillin, and 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 24 h at 25°C with shaking (220 rpm). Cells were harvested by centrifugation (4,600 × *g*, 15 min, 4°C). Cell pellets were resuspended in 50 mM Tris buffer, pH 8.0, and cells were disrupted by sonication (10 times for 30 s each at 10 μm with 30-s intervals) on ice. To obtain cell extracts, cell debris was removed by centrifugation (40,000 × *g*, 20 min, 4°C). Proteins were analyzed by SDS-PAGE analysis as described previously (24) and quantified using the Bradford method, using the Bio-Rad reagent and bovine serum albumin (BSA) as a standard.

Enzymatic activity of $FadD19_{DSM43269}$. The activity of $FadD19_{DSM43269}$ on various carboxylic acid sterols was analyzed by thin-layer chromatography (TLC). Reaction mixtures consisted of 20 mM Tris, pH 8.0, 5 mM $MgCl_2$, 0.5 mM substrate, and 2 mM ATP and/or 1 mM CoA, where applicable, in a final volume of 10 μl. Reactions were initiated by the addition of 1 μl of cell extract (CFE) containing $FadD19_{DSM43269}$ (~4 μg of total protein). Care was taken to use freshly produced enzyme from the same production batch in all experiments. CFE of *E. coli* BL21(DE3) carrying empty pET15b was used as a negative control. After 4 h of incubation at 30°C, total reaction volumes were directly applied to F_{254} silica gel TLC plates (Merck, Darmstadt, Germany) and separated using a mobile phase consisting of 1-butanol–acetic acid–water (80:25:40) for 30 min. Spots were visualized using Cer reagent consisting of 1% (wt/vol) $Ce(SO_4)_2$, 2.5% (wt/vol) $H_3[P(Mo_3O_{10})_4] \cdot H_2O$, and 8% (vol/vol) concentrated H_2SO_4 and developed by heating with a hot air gun.

Phylogenetic tree construction. Protein sequences of CoA ligases from *R. jostii* RHA1 were aligned using ClustalW; MEGA4.1 was used for phylogenetic tree construction (30). Amino acid sequences, annotated as acyl-CoA ligases or acyl-CoA synthetases, were obtained from the RHA1 genome website (www.rhodococcus.ca) and used for phylogenetic tree construction. Furthermore, BaiB of *Eubacterium* sp. strain VPI 12708 (accession number P19409) was included in the phylogenetic tree.

Nucleotide sequence accession numbers. DNA nucleotide sequencing was performed by Agowa (Berlin, Germany). The *R. rhodochrous* DSM43269 sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers HM588719 (pRESQ4690), HM588720 (pRESQ4693), HQ425873 (*kshA1*), HQ425874 (*kshA2*), HQ425875 (*kshA3*), HQ425876 (*kshA4*), and HQ425877 (*kshA5*).

RESULTS

Cloning and molecular characterization of a total of five *kshA* homologs from *Rhodococcus rhodochrous* DSM43269. We previously reported the cloning, heterologous expression, and characterization of the *kshA* gene of *R. rhodochrous* DSM43269 (22). In the current work, four additional *kshA* gene homologs from DSM43269 were cloned and sequenced as described in detail in Materials and Methods. Thus, a total of five *kshA* genes were identified in *R. rhodochrous* DSM43269. The gene homologs were designated *kshA1* through *kshA5* based on the similarities of the encoded KshA protein sequences to those of the three known *kshA* genes (*kshA1* to *kshA3*) of *R. erythropolis* SQ1 (33, 35). The previously reported KshA from DSM43269 (22) and the fifth KshA were less similar in amino acid sequence to KshA1, KshA2, or KshA3 of strain SQ1 than were the other newly identified KshA homologs of strain DSM43269 and were therefore renamed KshA4 and KshA5, respectively.

TABLE 3. Whole-cell bioconversion of sterols/steroids into ADD and 1,4-BNC by *R. rhodochrous* mutant strain RG32 after 72 h of incubation[a]

Steroid substrate	ADD (molar %)	1,4-BNC (%)
4-Androstene-3,17-dione	69 (±9)	NA
23,24-Bisnorchol-5-en-22-oic acid-3β-ol	–	60 (±21)
Campesterol	1 (±0)	49 (±9)
5-Cholenic acid-3β-ol	1 (±0)	77 (±4)
4-Cholestene-3-one	4 (±1)	79 (±2)
1,4-Cholestadiene-3-one	3 (±1)	71 (±4)
Cholesterol	3 (±1)	73 (±12)
β-Sitosterol	7 (±2)	67 (±7)

[a] NA, not applicable; –, not detected. The data represent the molar % conversion as averages of triplicate experiments; standard deviations are shown in parentheses.

Bioinformatic analysis revealed that the typical Rieske Fe_2S_2 binding domain (C-X-H-$X_{16,17}$-C-X_2-H) and the nonheme Fe^{2+} motif (D-X_3-D-X_2-H-X_4-H) (35) were present in all five KshA homologs.

Construction and characterization of *kshA* null mutant strain *R. rhodochrous* RG32 performing selective sterol side chain degradation. The cloned DNA fragments of wild-type *R. rhodochrous* DSM43269 were used to construct a 5-fold *kshA* null mutant, designated strain RG32 (see Materials and Methods). Whole-cell biotransformations of 4-androstene-3,17-dione (AD, Fig. 1, compound VIII) by strain RG32 resulted in 69% molar conversion into 1,4-androstadiene-3,17-dione (ADD, Fig. 1, compound VI) (Table 3 and Fig. 1C, KSTD activity), which was not accumulated in detectable amounts by wild-type strain DSM43269 (data not shown), confirming impaired KSH activity (35). Next, mutant strain RG32 was tested in whole-cell biotransformations for its ability to perform selective side chain degradation on a range of sterols/steroids. All compounds tested were converted into ADD (Fig. 1, compound VI) and 3-oxo-23,24-bisnorchola-1,4-dien-22-oic acid (1,4-BNC; Fig. 1, compound V) in molar ratios of 1 to 7% and 50 to 80%, respectively, depending on the substrate used (Table 3). Thus, mutant strain RG32 is completely blocked in steroid ring degradation and capable of selective sterol side chain degradation, enabling analysis of putative sterol side chain degradation genes.

Cloning homologous cholesterol catabolic genes from *R. rhodochrous* DSM43269. Sequence data on the cholesterol catabolic gene cluster of *R. rhodochrous* DSM43269, necessary to perform mutational analysis of genes involved in sterol side chain degradation in strain RG32, were not available yet. Therefore, homologs of *R. jostii* RHA1 genes predicted to be involved in sterol side chain degradation were cloned from strain DSM43269. First, a genomic library of strain DSM43269 (22) was screened using degenerate PCR primers based on amino acid sequences that were highly conserved among actinobacterial homologs of strain RHA1 Ro04690 and FadE26 (Ro04693) (Fig. 2A). Library screening by PCR resulted in the isolation of two separate clones that were sequenced and analyzed (Fig. 2B and C). A 5.4-kb genomic fragment (Fig. 2B) carried *$ro04690_{DSM43269}$*, encoding a protein displaying 85%

TABLE 4. Whole-cell bioconversion of sterols by *R. rhodochrous* RG32 and mutants thereof into ADD and 1,4-BNC, measured after 72 h of incubation[a]

Strain	% conversion of sterol:					
	Cholesterol		β-Sitosterol		Campesterol	
	ADD	1,4-BNC	ADD	1,4-BNC	ADD	1,4-BNC
RG32	3 (±1)	73 (±12)	7 (±2)	67 (±7)	1 (±0)	49 (±9)
RG32Δ*ro04690*$_{DSM43269}$	4 (±1)	74 (±8)	7 (±4)	54 (±9)	ND	ND
RG32Δ*ro04690*$_{DSM43269}$ Δ*fadE26*	3 (±1)	72 (±4)	6 (4)	61 (±9)	ND	ND
RG32Δ*ro04690*$_{DSM43269}$ Δ*fadE26* Ω*fadE27*	3 (±2)	70 (±6)	7 (±2)	65 (±6)	ND	ND
RG32Ω*fadD17*	3 (±2)	74 (±9)	8 (±2)	64 (±5)	ND	ND
RG32Δ*fadD19*	6 (±2)	64 (±4)	−	−	−	−
RG32Δ*fadD19*+pCOMP*fadD19*$_{DSM43269}$	ND	ND	6 (±1)	60 (±4)	ND	ND
RG32Δ*fadD19* Ω*fadD17*	6 (±2)	69 (±1)	−	−	ND	ND

[a] ND, not determined; −, not detected. The data represent averages of duplicate experiments; standard deviations are shown in parentheses.

amino acid sequence identity with Ro04690$_{RHA1}$. Two genes located immediately upstream of *ro04690*$_{DSM43269}$ encode proteins sharing 74% and 76% amino acid sequence identity with the deduced amino acid sequences of *echA19* (partial sequence) and *fadD19* in strain RHA1, respectively. Three genes located downstream of *ro04690*$_{DSM43269}$ encode proteins showing the highest similarities to Ro03510 (23%), Ro04422 (25%), and Ro01580 (56%) of strain RHA1, respectively. Bioinformatic analysis of the sequence of a 5.5-kb insert (Fig. 2C) confirmed the presence of *fadE26*$_{DSM43269}$, whose gene product displayed 86% amino acid sequence identity to its counterpart in strain RHA1. Moreover, the fragment contained genes that encode proteins with 46 to 70% amino acid sequence identities to *fadD17* (partial sequence), *fadE27*, *fdxD*, and *hsd4A* of strain RHA1, exhibiting identical genetic organizations (Fig. 2). The two cloned genomic fragments of strain DSM43269 did not show sequence overlap.

FadD19 is essential for C-24 branched-chain sterol side chain degradation. To substantiate a role of *fadD17*$_{DSM43269}$, *fadD19*$_{DSM43269}$, *fadE26*$_{DSM43269}$, *fadE27*$_{DSM43269}$, and *ro04690*$_{DSM43269}$ in sterol side chain degradation, gene inactivation mutants of strain RG32 were constructed. Biotransformations of cholesterol with whole-cell cultures of these mutants revealed that all mutants displayed parental phenotypes, with similar amounts of ADD and 1,4-BNC accumulating from cholesterol as observed with parent strain RG32 (Table 4). Although the putative CoA ligases encoded by *fadD17* and *fadD19* show only a relatively low sequence identity at the amino acid level (21 and 23% for strain RHA1 and strain DSM43269, respectively), the possibility that they are functional homologs able to cross-complement each other during cholesterol catabolism could not be ruled out. Therefore, a Ω*fadD17* inactivation mutant was constructed in strain RG32Δ*fadD19* and its ability to degrade cholesterol was tested by whole-cell biotransformation. Cholesterol degradation also was unaffected in RG32Δ*fadD19* Ω*fadD17*, displaying conversion rates similar to that of strain RG32 (Table 4).

FadE26, FadE27, and Ro04690 all belong to the superfamily of acyl-CoA dehydrogenases (7). We therefore also constructed a double gene inactivated mutant strain, RG32Δ*ro04690*$_{DSM43269}$ Δ*fadE26*, and a triple mutant, RG32Δ*ro04690*$_{DSM43269}$ Δ*fadE26* Ω*fadE27*, to prevent possible cross-complementation. However, all these mutants were unaffected in cholesterol side chain degradation and displayed parent strain RG32 phenotypes (Table 4). Although clearly upregulated in strain RHA1 cells grown on cholesterol (Fig. 2A), either these *R. rhodochrous* DSM42369 genes are not involved in cholesterol side chain degradation or the reaction can be performed by additional isoenzymes encoded by strain DSM42369.

Next, all mutants were tested in whole-cell biotransformations with β-sitosterol, a C-24–ethyl branched phytosterol (Fig. 1B). Interestingly, strain RG32Δ*fadD19* was blocked in its ability to convert the side chain of β-sitosterol (Table 4). All other mutants displayed parental strain phenotypes, accumulating similar amounts of ADD and 1,4-BNC from β-sitosterol as did strain RG32 (Table 4). Further analysis of the RG32Δ*fadD19* mutant revealed that also the degradation of campesterol, a C-24–methyl branched sterol, was blocked (Table 4).

When incubated with β-sitosterol, strain RG32Δ*fadD19* accumulated metabolites that were not detected in strain RG32 (Fig. 3). These metabolites corresponded to ring-oxidized derivatives of the β-sitosterol mixture, i.e., 4-sitostene-3-one and 4-campestene-3-one, as was confirmed by reference samples. Mutant strain RG32Δ*fadD19* regained parental phenotype when complemented with *fadD19*$_{DSM43269}$ under the control of the *aphII* promoter, with β-sitosterol as the substrate (Table 4). This excluded the possibility that β-sitosterol side chain degradation in RG32Δ*fadD19* was blocked by polar effects rather than by inactivation of *fadD19* directly.

FadD19$_{DSM43269}$ displays sterol-CoA ligase activity toward C-26-oic acid steroids. The gene inactivation studies in strain RG32 showed that *fadD19* is essential for side chain degradation of branched-chain sterols. To substantiate that *fadD19* encodes a steroid-CoA ligase, FadD19$_{DSM43269}$ activity was tested. Heterologous expression of the protein was achieved using *E. coli* strain BL21(DE3) carrying pET15b*fadD19*$_{DSM43269}$. Cell extracts (CFE) derived from *E. coli* cultures expressing FadD19$_{DSM43269}$ were active toward 5-cholestene-26-oic acid-3β-ol, using ATP and CoA as cosubstrates (Fig. 4A). Since CoA ligases require the presence of a carboxylic acid for their activity, FadD19$_{DSM43269}$ must have been active toward the side chain of 5-cholestene-26-oic acid-3β-ol. A negative control, consisting of the same components but with CFE obtained from cultures of the same *E. coli* strain carrying empty pET15b vector, did not show product formation (Fig. 4B). Incubations of CFE containing FadD19$_{DSM43269}$ also resulted in the for-

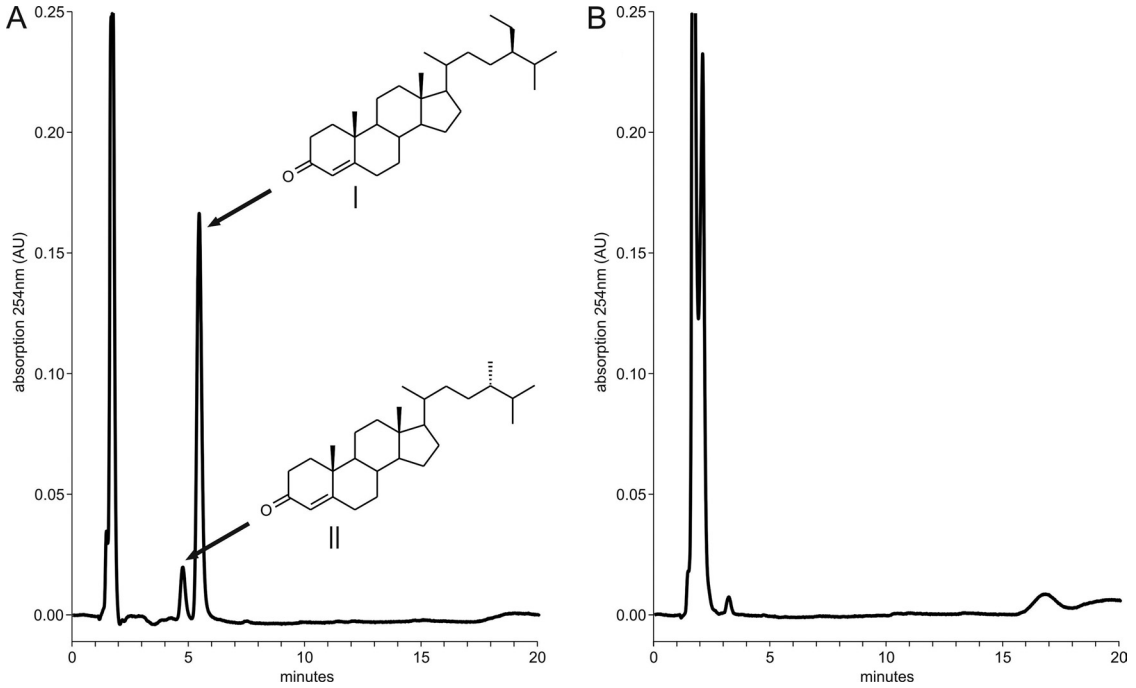

FIG. 3. HPLC graphs of steroid extracts from β-sitosterol bioconversions after 3 days of incubation. (A) Profile of mutant strain RG32Δ*fadD19* showing the accumulation of metabolites (degradation pathway intermediates) that, based on identical HPLC retention times, were identified as 4-sitostene-3-one (I) and 4-campestene-3-one (II). (B) Profile of parent strain RG32.

mation of reaction products with 3-oxo-4-cholestene-26-oic acid or 5-cholenic acid-3β-ol in a steroid substrate-, ATP-, and CoA-dependent manner (Fig. 4A). Omitting any of these reaction components resulted in loss of enzyme activity, indicating that these are all essential components. Therefore, the products formed are most likely the CoA esters of 5-cholestene-26-oic acid-3β-ol and 3-oxo-4-cholestene-26-oic acid, confirming that $FadD19_{DSM43269}$ functions as a steroid-CoA

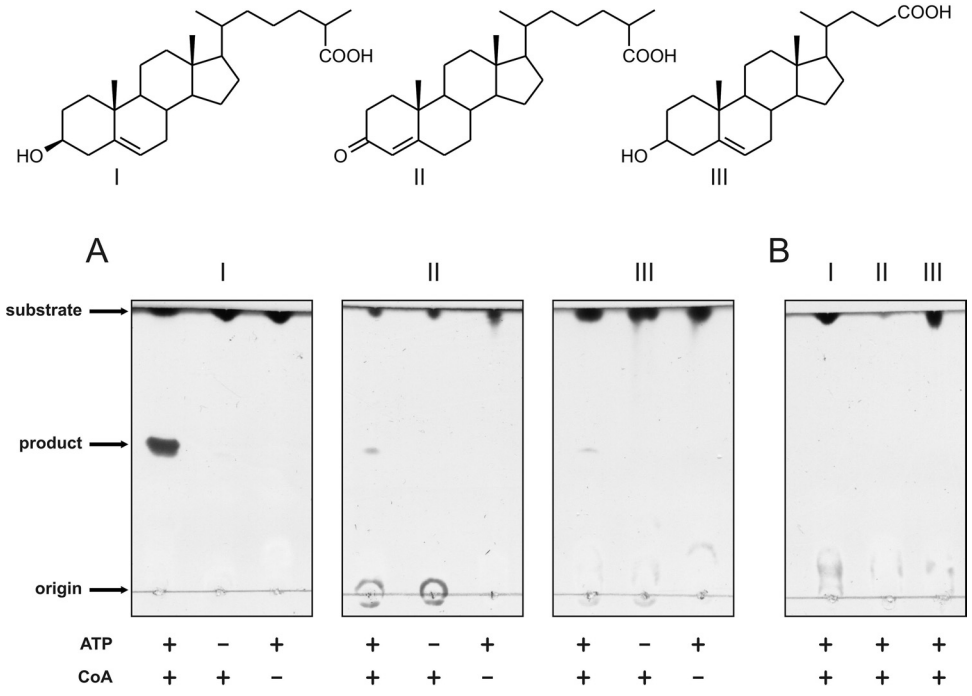

FIG. 4. (A) TLC analysis of the reactions of cell extracts (CFE) of *E. coli* BL21(DE3) cells expressing $fadD19_{DSM43269}$, incubated with 5-cholestene-26-oic acid-3β-ol (I), 3-oxo-4-cholestene-26-oic acid (II), and 5-cholenic acid-3β-ol (III). (B) TLC analysis of reactions of CFE of BL21 cells containing empty plasmid pET15b, using the same sterol substrates (I to III). All incubations were performed for 4 h at 30°C and contained the cofactor Mg^{2+}. ATP and CoA were either included (+) or omitted (−) as a negative control.

ligase. Activity toward C-24 branched-chain steroids with a terminal carboxylic acid could not be tested, since these compounds were not (commercially) available.

Bioinformatic analysis and phylogeny of *R. jostii* RHA1 CoA ligases. Database searches served to identify homologs of both $FadD19_{RHA1}$ and $FadD17_{RHA1}$ in closely related sterol-degrading actinobacteria, including *Nocardia farcinica* IFM 10152 (Nfa5290 and Nfa24170; with 75 and 47% identity, respectively), *Mycobacterium smegmatis* strain MC2 155 (MSMEG_5914 and MSMEG_5908; with 67 and 57% identity, respectively), *M. tuberculosis* H37Rv (Rv3515c and Rv3506; with 67 and 56% identity, respectively), and *Rhodococcus equi* 103S and ATCC 33707 (HMPREF0724_4865 and HMPREF0724_4863; with 77 and 72% identity, respectively). A comparison of $FadD19_{RHA1}$ and $FadD17_{RHA1}$ with the amino acid sequence of the cholic acid-CoA ligase, encoded by *baiB*, revealed relatively low similarities (24 and 20% identity, respectively). All sequences in the strain RHA1 genome annotated as CoA ligase or CoA synthetase were compared and depicted in a phylogenetic tree (see Fig. S1 in the supplemental material). This revealed that the strain RHA1 genome contains closely related homologs of $FadD17_{RHA1}$ and $FadD19_{RHA1}$, i.e., Ro05822 (44% identity) and Ro04675 (49% identity), respectively. These putative enzymes thus may have a similar steroid-CoA ligase activity, resulting in a metabolic redundancy for activation of the side chain of 5-cholestene-26-oic acid-3β-ol metabolites.

DISCUSSION

This study identified FadD19 *of R. rhodochrous* DSM43269 as a steroid-CoA ligase, essential for the degradation of C-24 branched-chain sterols *in vivo*. An unmarked *fadD19* deletion mutant in *kshA* null mutant strain RG32 was blocked in side chain degradation of the C-24 branched sterols β-sitosterol and campesterol but not cholesterol (Table 4). In addition, mutant RG32Δ*fadD19* accumulated ring-oxidized derivatives from β-sitosterol (Fig. 3), indicating that in this mutant no sterol side chain degradation occurred, but only steroid ring oxidation. From these results, we conclude that $FadD19_{DSM43269}$ catalyzes an essential step in the side chain degradation of C-24 branched sterols in *R. rhodochrous* DSM43269.

In *R. jostii* RHA1, *fadD17* is located proximal to *fadD19* within the cholesterol catabolic gene cluster and highly upregulated during growth on cholesterol. Thus, *fadD17* is a likely candidate to encode a steroid-CoA ligase involved in cholesterol side chain activation (36). Mutant strains with an inactivated $fadD17_{DSM43269}$ gene in both RG32 and RG32Δ*fadD19*, however, were still capable of transformation of the cholesterol side chain. Therefore, it remains elusive whether side chain activation during cholesterol catabolism is performed by multiple redundant CoA ligases or whether a yet-unidentified, highly specific enzyme catalyzes this reaction *in vivo*.

Direct enzyme activity measurements showed that $FadD19_{DSM43269}$ was able to activate the side chains of various steroid carboxylic acids (Fig. 4). The intensities of the reaction product spots suggest that $FadD19_{DSM43269}$ has substrate preference for steroids possessing longer side chains over those with shorter ones. Furthermore, substrates with a 3-hydroxy-5-ene ring structure may be better substrates than their oxidized forms. However, it is difficult to quantitatively compare the reaction products due to the discrepancy in spot intensity between the 3-hydroxy-5-ene and 3-oxo-4-ene substrates at the same substrate concentration. Additional biochemical studies are thus required to assess the substrate specificity of $FadD19_{DSM43269}$.

Despite the fact that $FadD19_{DSM43269}$ was able to activate 5-cholestene-26-oic acid-3β-ol and derivatives thereof *in vitro*, deletion of $fadD19_{DSM43269}$ in strain RG32 did not affect cholesterol degradation. Therefore, it is most plausible that, besides *fadD19* and possibly *fadD17*, other isoenzymes are encoded by the strain DSM43269 genome that can complement 5-cholestene-26-oic acid-3β-ol activation in this mutant. Orthologs of Ro05822 and Ro04675 (see Fig. S1 in the supplemental material) may be present in strain DSM43269 and could account for the lack of phenotype found with our mutants in whole-cell biotransformations with cholesterol. CoA ligases are known to be promiscuous enzymes, which often have overlapping substrate specificities (3, 21), further supporting the hypothesis that the genomes of strain RHA1 and strain DSM43269 may encode multiple enzymes with the ability to activate the side chain of 5-cholestene-26-oic acid-3β-ol or derivatives thereof (e.g., its 3-oxo-4-ene metabolite).

Similarly, inactivation of multiple genes encoding putative acyl-CoA dehydrogenases (i.e., $fadE26_{DSM43269}$, $fadE27_{DSM43269}$, and $ro04690_{DSM43269}$) in strain RG32 did not hamper cholesterol nor β-sitosterol side chain degradation (Table 4). Rhodococcal genomes are very rich in genes encoding β-oxidation enzymes, making it very likely that enzymatic redundancy for these functions exists. However, the possibility that the $fadE26_{DSM43269}$, $fadE27_{DSM43269}$, and $ro04690_{DSM43269}$ genes in fact do not have a role in sterol side chain degradation cannot be ruled out at the moment.

Interestingly, there is apparently no redundancy for activation of C-24 branched sterol side chains, since deletion of $fadD19_{DSM4269}$ alone in strain RG32 resulted in impaired degradation of C-24 branched-chain sterols. $FadD19_{DSM43269}$ may be the only enzyme able to activate the side chains of C-24 branched phytosterols, which possess a bulkier side chain than that of cholesterol. However, it cannot be excluded that a homolog (e.g., Ro04675) is encoded by strain DSM43269 that is able to catalyze the same reaction but that it is not expressed in mutant RG32Δ*fadD19* under the conditions used.

The results described in our current study are consistent with FadD19 of *R. rhodochrous* DSM43269 performing a steroid-CoA ligase reaction with an *in vivo* role in the degradation of C-24 branched sterol side chains. $FadD19_{H37Rv}$ of the human pathogen *M. tuberculosis* H37Rv, encoded by *rv3515c*, possesses CoA ligase activity and catalyzes the formation of acyl-CoA from long-chain fatty acids (31). BLAST searches with $FadD19_{DSM43269}$ and $FadD19_{RHA1}$ against the H37Rv genome showed that they both have the highest sequence identity with $FadD19_{H37Rv}$; $FadD19_{RHA1}$ and $FadD19_{H37Rv}$ also were best reciprocal hits. The high similarity (67%) between these enzymes suggests an *in vivo* role for $FadD19_{H37Rv}$ in sterol metabolism of *M. tuberculosis* H37Rv.

The study furthermore described the construction of mutant strain *R. rhodochrous* RG32, blocked in sterol ring degradation, and demonstrated its successful use as a tool to study the process of microbial sterol side chain degradation specifically,

allowing the future characterization of other putative sterol side chain degradation genes.

ACKNOWLEDGMENTS

This work was financially supported by grants of the IBOS (M.H.W.) and B-Basic (M.P.) programs of ACTS (Advanced Chemical Technologies for Sustainability), NWO (Netherlands Organization for Scientific Research).

REFERENCES

1. **Arima, K., M. Nagasawa, M. Bae, and G. Tamura.** 1969. Microbial transformation of sterols. Part I. Decomposition of cholesterol by microorganisms. Agric. Biol. Chem. **33:**1636–1643.
2. **Arima, K., T. Nakamatsu, and T. Beppu.** 1978. Microbial production of 3-oxobisnorchola-1,4-dien-22-oic acid. Agric. Biol. Chem. **42:**411–416.
3. **Arora, P., A. Vats, P. Saxena, D. Mohanty, and R. S. Gokhale.** 2005. Promiscuous fatty acyl CoA ligases produce acyl-CoA and acyl-SNAC precursors for polyketide biosynthesis. J. Am. Chem. Soc. **127:**9388–9389.
4. **Birkenmaier, A., et al.** 2007. Biochemical and genetic investigation of initial reactions in aerobic degradation of the bile acid cholate in *Pseudomonas* sp. strain Chol1. J. Bacteriol. **189:**7165–7173.
5. **Capyk, J. K., et al.** 2009. Mycobacterial cytochrome P450 125 (CYP125) catalyzes the terminal hydroxylation of C27 steroids. J. Biol. Chem. **284:** 35534–35542.
6. **Chen, C. S.** 1985. Ph.D. thesis. University of Wisconsin—Madison, Madison, WI.
7. **Daubner, S. C., G. Gadda, M. P. Valley, and P. F. Fitzpatrick.** 2002. Cloning of nitroalkane oxidase from *Fusarium oxysporum* identifies a new member of the acyl-CoA dehydrogenase superfamily. Proc. Natl. Acad. Sci. U. S. A. **99:**2702–2707.
8. **Fernandes, P. J., J. A. Powell, and J. A. Archer.** 2001. Construction of *Rhodococcus* random mutagenesis libraries using Tn5 transposition complexes. Microbiology **147:**2529–2536.
9. **Fujimoto, Y., C. S. Chen, Z. Szeleczky, D. Ditullio, and C. J. Sih.** 1982. Microbial degradation of the phytosterol side chain. I. Enzymic conversion of 3-oxo-24-ethylcholest-4-en-26-oic acid into 3-oxochol-4-en-24-oic acid and androst-4-ene-3,17-dione. J. Am. Chem. Soc. **104:**4718–4720.
10. **Fujimoto, Y., C. S. Chen, A. S. Gopalan, and C. J. Sih.** 1982. Microbial degradation of the phytosterol side chain. II. Incorporation of $NaH^{14}CO_3$ onto the C-28 position. J. Am. Chem. Soc. **104:**4720–4722.
11. **Knol, J., K. Bodewits, G. I. Hessels, L. Dijkhuizen, and R. R. van der Geize.** 2008. 3-Keto-5α-steroid delta1-dehydrogenase from *Rhodococcus erythropolis* SQ1 and its orthologue in *Mycobacterium tuberculosis* H37Rv are highly specific enzymes that function in cholesterol catabolism. Biochem. J. **410:** 339–346.
12. **Mallonee, D. H., J. L. Adams, and P. B. Hylemon.** 1992. The bile acid-inducible *baiB* gene from *Eubacterium* sp. strain VPI 12708 encodes a bile acid-coenzyme A ligase. J. Bacteriol. **174:**2065–2071.
13. **Marsheck, W. J., S. Kraychy, and R. D. Muir.** 1972. Microbial degradation of sterols. Appl. Microbiol. **23:**72–77.
14. **Martin, C. K. A., and F. Wagner.** 1976. Microbial transformation of β-sitosterol by *Nocardia* sp. M29. Eur. J. Appl. Microbiol. **2:**243–255.
15. **McLean, K. J., et al.** 2009. The structure of *Mycobacterium tuberculosis* CYP125: molecular basis for cholesterol binding in a P450 needed for host infection. J. Biol. Chem. **284:**35524–35533.
16. **McLeod, M. P., et al.** 2006. The complete genome of *Rhodococcus* sp. RHA1 provides insights into a catabolic powerhouse. Proc. Natl. Acad. Sci. U. S. A. **103:**15582–15587.
17. **Mohn, W. W., et al.** 2008. The actinobacterial *mce4* locus encodes a steroid transporter. J. Biol. Chem. **283:**35368–35374.
18. **Nesbitt, N. M., et al.** 2010. A thiolase of *Mycobacterium tuberculosis* is required for virulence and production of androstenedione and androstadienedione from cholesterol. Infect. Immun. **78:**275–282.
19. **Ouellet, H., et al.** 2010. *Mycobacterium tuberculosis* CYP125A1, a steroid C27 monooxygenase that detoxifies intracellularly generated cholest-4-en-3-one. Mol. Microbiol. **77:**730–742.
20. **Pandey, A. K., and C. M. Sassetti.** 2008. Mycobacterial persistence requires the utilization of host cholesterol. Proc. Natl. Acad. Sci. U. S. A. **105:**4376–4380.
21. **Pei, Z., et al.** 2003. The acyl-CoA synthetase "bubblegum" (lipidosin): further characterization and role in neuronal fatty acid beta-oxidation. J. Biol. Chem. **278:**47070–47078.
22. **Petrusma, M., L. Dijkhuizen, and R. van der Geize.** 2009. *Rhodococcus rhodochrous* DSM 43269 3-ketosteroid 9α-hydroxylase, a two-component iron-sulfur-containing monooxygenase with subtle steroid substrate specificity. Appl. Environ. Microbiol. **75:**5300–5307.
23. **Rosłoniec, K. Z., et al.** 2009. Cytochrome P450 125 (CYP125) catalyzes C26-hydroxylation to initiate sterol side chain degradation in *Rhodococcus jostii* RHA1. Mol. Microbiol. **74:**1031–1043.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. **Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
26. **Sih, C. J.** 1962. Mechanisms of steroid oxidation by microorganisms. Biochim. Biophys. Acta **62:**541–547.
27. **Sih, C. J., H. H. Tai, Y. Y. Tsong, S. S. Lee, and R. G. Coombe.** 1968. Mechanisms of steroid oxidation by microorganisms. XIV. Pathway of cholesterol side-chain degradation. Biochemistry **7:**808–818.
28. **Sih, C. J., K. C. Wang, and H. H. Tai.** 1968. Mechanism of steroid oxidation by microorganisms. XIII. C22 acid intermediates in the degradation of the cholesterol side chain. Biochemistry **7:**796–807.
29. **Szentirmai, A.** 1990. Microbial physiology of sidechain degradation of sterols. J. Ind. Microbiol. **6:**101–116.
30. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
31. **Trivedi, O. A., et al.** 2004. Enzymic activation and transfer of fatty acids as acyl-adenylates in mycobacteria. Nature **428:**441–445.
32. **van der Geize, R., et al.** 2008. A novel method to generate unmarked gene deletions in the intracellular pathogen *Rhodococcus equi* using 5-fluorocytosine conditional lethality. Nucleic Acids Res. **36:**e151.
33. **van der Geize, R., G. I. Hessels, M. Nienhuis-Kuiper, and L. Dijkhuizen.** 2008. Characterization of a second *Rhodococcus erythropolis* SQ1 3-ketosteroid 9α-hydroxylase activity comprising a terminal oxygenase homologue, KshA2, active with oxygenase-reductase component KshB. Appl. Environ. Microbiol. **74:**7197–7203.
34. **van der Geize, R., G. I. Hessels, R. van Gerwen, P. van der Meijden, and L. Dijkhuizen.** 2001. Unmarked gene deletion mutagenesis of kstD, encoding 3-ketosteroid Δ1-dehydrogenase, in *Rhodococcus erythropolis* SQ1 using sacB as counter-selectable marker. FEMS Microbiol. Lett. **205:**197–202.
35. **van der Geize, R., G. I. Hessels, R. Van Gerwen, P. Van der Meijden, and L. Dijkhuizen.** 2002. Molecular and functional characterization of *kshA* and *kshB*, encoding two components of 3-ketosteroid 9α-hydroxylase, a class IA monooxygenase, in *Rhodococcus erythropolis* strain SQ1. Mol. Microbiol. **45:**1007–1018.
36. **van der Geize, R., et al.** 2007. A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *Mycobacterium tuberculosis* survival in macrophages. Proc. Natl. Acad. Sci. U. S. A. **104:**1947–1952.
37. **Watanabe, K., H. Aihara, N. Tachi, and R. Nakamura.** 1987. Degradations of 4-cholesten-3-one and 1,4-androstadiene-3,17-dione by cholesterol-degrading bacteria. J. Appl. Bacteriol. **62:**151–155.